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THE SUBCELLULAR DISTRIBUTIONS OF SOME HYDROLYTIC ENZYMES IN UNFERTILIZED EGGS OF THE SEA URCHIN, ARBACIA PUNCTULATA

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The identification of specific cellular granules containing hydrolytic enzymes was first made by de Duve and co-workers (1955) by centrifugal fractionation of rat-liver homogenates. The name lysosome was proposed at this time for these granules because several distinct acid hydrolases appeared to be located within them and to be released in a paralleled manner in preparations subjected to disruptive treatments such as freezing and thawing. At least 12 hydrolytic enzymes showing an acid pH optimum are presently believed to be associated with the lysosome. These include ribonuclease, acid deoxyribonuclease, acid phosphatase, phosphoprotein phosphatase, cathepsin, collagenase, *alpha*-glucosidase, *beta*-N-acetylglucosaminidase, *beta*-glucuronidase, *alpha*-mannosidase, and aryl-sulfatase (*cf.* review by Novikoff, 1961). Although most of the work on the distribution of hydrolytic enzymes has been with adult mammalian tissue, investigations of acid hydrolases have also been extended to some invertebrates. In most of these, however, only brief surveys have been made of characteristic enzymes, and no attempts to isolate any specific granules have been reported (*cf.* review by de Reuck and Cameron, 1963).

Lysosomal granules have been implicated in a few developmental processes involving regression and resorption of embryonic cells, especially in Mullerian duct rudiments of male chick embryos (Scheib-Pfleger and Wattiaux, 1962) and in the tails of amphibian tadpoles undergoing metamorphosis (Weber, 1963). In the rat egg Dalcq (1963) has observed granules in which high acid phosphatase was demonstrated cytochemically and which stained metachromatically. The metachromatic granules observed in invertebrate eggs appear to be of two types, designated as *alpha* and *beta* granules by Pasteels and Mulnard (1957), who concluded that the larger *beta* granules were not stained directly but received dye from the smaller *alpha* granules. These investigators found by centrifugation that the *alpha* granules are concentrated near the centrifugal extremity of the egg or blastomeres, while the

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beta granules are sedimented in the hyaline part of the cytoplasm. Dalcq (1963) has recently concluded from comparative electron microscopic and cytochemical investigations of early developmental stages that the yolk platelets are a source not only of nutrient material but also of definite organelles with phosphatase activity. Pasteels and de Harven (1963) confirmed this by a series of electron microscope findings which demonstrated the transformation of yolk platelets into microvesicular bodies similar to the metachromatic granules observed in the living eggs, and the rupture of the microvesicular bodies to release minute phosphohydrolase granules. The only resemblances these granules have to lysosomes, however, are their apparent ability to rupture, and their possession of acid phosphatase.

Attempts to identify specific acid hydrolase granules in embryonic tissue have usually been made only by means of electron microscopy and cytochemistry. Dalcq (1963) proposed at a recent symposium that the most direct approach to determining the presence or absence of particles containing specific hydrolases would be the application of density gradient centrifugation to homogenates of eggs at various stages, to determine whether a layer of particles containing an array of lytic enzymes could be isolated. This present investigation is concerned with the problem of attempting to isolate a fraction containing an appreciable concentration of acid hydrolases from homogenates of unfertilized eggs of *Arbacia punctulata.* To determine the distribution within the subcellular fractions differential centrifugation has been applied to sucrose homogenates of both eggs and adult gut tissue. Isolation of specific sets of granules containing acid hydrolase activity has been attempted only on the large visible-granule fraction of the egg homogenate, by centrifugation on sucrose density gradients. The preliminary results obtained by the differential and density gradient centrifugation indicate the probable existence in the egg of more than one type of large granule possessing hydrolytic enzymes.

Materials and Methods

Biolological procedures

The animals used in this study, *Arbacia punctulata,* were collected along the Eastern Shore region of the Chesapeake Bay. They were stored in the laboratory at 23° C. in aerated, polyethylene aquaria filled with artificial sea water at 33‰ made from Utility Seven-Seas Marine Mix (Utility Chemical Company, Patterson, New Jersey). In order to obtain adult tissue, the entire gut was removed by dissection, placed in cold, artificial sea water, blotted carefully, and weighed. Eggs were obtained by electrically inducing shedding in the females, using alternating current at 30 volts (Harvey, 1954). The eggs were washed three times by settling in artificial sea water prepared from reagent grade salts and distilled water. To remove the jelly coat, the eggs were treated with acid sea water at pH 4.6–4.8 and allowed to settle (Allen, 1957). The eggs were washed an additional time in sea water buffered at pH 8.0 with 0.02 *M* tris-(hydroxymethyl)aminomethane (hereafter referred to as "tris") and packed in a hand centrifuge to prepare them for homogenization.

Adult gut and eggs were prepared for fractionation by the same methods. A 10% homogenate of each tissue was made, based on a weight-volume ratio for the gut tissues and on a volume/volume ratio in the eggs. The tissues were homog-

enized at 0° C. in a hand-operated Tenbroeck glass homogenizer in 0.98 *M* sucrose containing 10^{-3} *M* ethylenediamine tetraacetic acid (EDTA) and buffered at pH 7.5 with 0.05*M* tris. This buffered sucrose was used for suspending all of the cell fractions obtained by later centrifugation. A starting material which was essentially nuclei-free was prepared, based on the procedure of Berthet and de Duve (1951) with modifications to allow for the differences in sucrose density. The 10% homogenate was centrifuged for ten minutes at 2000 rpm for the gut homogenate and at 500 rpm for the egg homogenate, in rotor No. 253 in an International PR-2 centrifuge at 0° C. The egg homogenate was centrifuged at the much lower speed to remove the debris and whole cells without removing the majority of heavy granules. The supernatant fluid was then decanted and saved. The sedimented fraction from each tissue was rehomogenized in an additional 3 to 5 ml. of buffered 0.98 *M* sucrose-EDTA and recentrifuged at the same speed as before. The combined supernatant fluids were used for the subsequent isolation of granules. The final precipitate containing clumped nuclei, cell fragments, and whole cells was discarded.

The various subcellular fractions were obtained by a modification of the technique used by Applemans, Wattiaux and de Duve (1955). The procedure is outlined in the flow sheet given below. All of the operations described were carried out at or near 0° C. The preparations were either used immediately or frozen at −18° C. for up to 48 hours. Tests of the effects of freezing on enzyme activities were made on whole homogenates. None of the enzymes for which data are reported were decreased in activity as a result of freezing.

Fraction I from eggs was centrifuged on a layered sucrose gradient to separate particles of different densities (de Duve, Berthet and Beaufay, 1959). The separated granule layers were removed by pipetting from the top or by puncturing the bottom of the tube and allowing the sucrose to drip out slowly.

The staining properties of the granules obtained on the density gradient were examined by dividing Fraction I into three equal portions. One tube served as a control; the other two contained 10 to 15 drops of 0.1% dye in a total volume of 5 ml. After an initial centrifugation at 17,500 times gravity to remove the excess stain, the stained preparations were placed over the same density gradients as above, and centrifuged for one hour at 90,000 times gravity.

Chemical procedures

Determinations of protein, nucleic acid, acid phosphatase, esterase, lipase, arylsulfatase, *beta*-galactosidase, ribonuclease (RNAase), and proteolytic activity were attempted according to the methods outlined below. All enzyme reactions were run at 25° C. Tests for linearity were made on whole homogenates in preliminary experiments.

Before determinations of protein and nucleic acid were made, the samples were extracted according to the method of Schmidt and Thannhauser (1945) three times with cold 10% W/V trichloroacetic acid (TCA), twice with boiling ethanol-ether (3:1 V/V) and twice with hot 5% TCA at 90° for 15 minutes. The hot TCA extracts were combined and used for nucleic acid determination by the ultraviolet absorption procedure of Schneider (1957). The protein was suspended in 1 N sodium hydroxide and determined with Folin-Ciocalteau reagent (Fisher Chemical

Company) by the method of Lowry *et al.* (1951). Standard absorbance curves were prepared, using solutions of reagent grade RNA and of crystalline bovine serum albumin, both obtained from Nutritional Biochemicals Corporation.

Assays of acid phosphatase, esterase, lipase, aryl-sulfatase and *beta*-galactosidase were attempted by using as substrates *p*-nitrophenyl phosphate, acetate, stearate, sulfate and galactoside, respectively. All substrates were obtained from Nutritional Biochemicals Corporation, except for *p*-nitrophenyl phosphate, and *p*-nitrophenyl-β-D galactoside, which were obtained from the Sigma Chemical Company. The *p*-nitrophenol liberated from each substrate was determined in alkaline solution (except as noted below) at 400 mμ on the Beckman DU spectrophotometer, or with filter No. 42 on the Klett-Summerson colorimeter. The details of each procedure are presented below.

Acid phosphatase was determined by the method of Burch *et al.* (1952). Esterase was assayed at pH 7.1 by the method of Huggins and Laprides (1947), using a standard curve of *p*-nitrophenol at the same pH. The method used for aryl-sulfatase depends on the rather small change in absorbance at 400 mμ which occurs when *p*-nitrophenyl sulfate and enzyme are incubated at pH 5.1. The reaction mixture contained 2.0 mg. of substrate, 0.03 *M* sodium acetate, pH 5.0 and 0.05 to 0.15 ml. of enzyme in a total volume of 3.0 ml. Readings were taken for 15 minutes on the DU or DB spectrophotometer against a blank containing buffer and enzyme. Spontaneous hydrolysis of the substrate was negligible at this pH. A standard curve was prepared from *p*-nitrophenol at pH 5.1. Ribonuclease was measured by the method of Dubos and Thompson (1938).

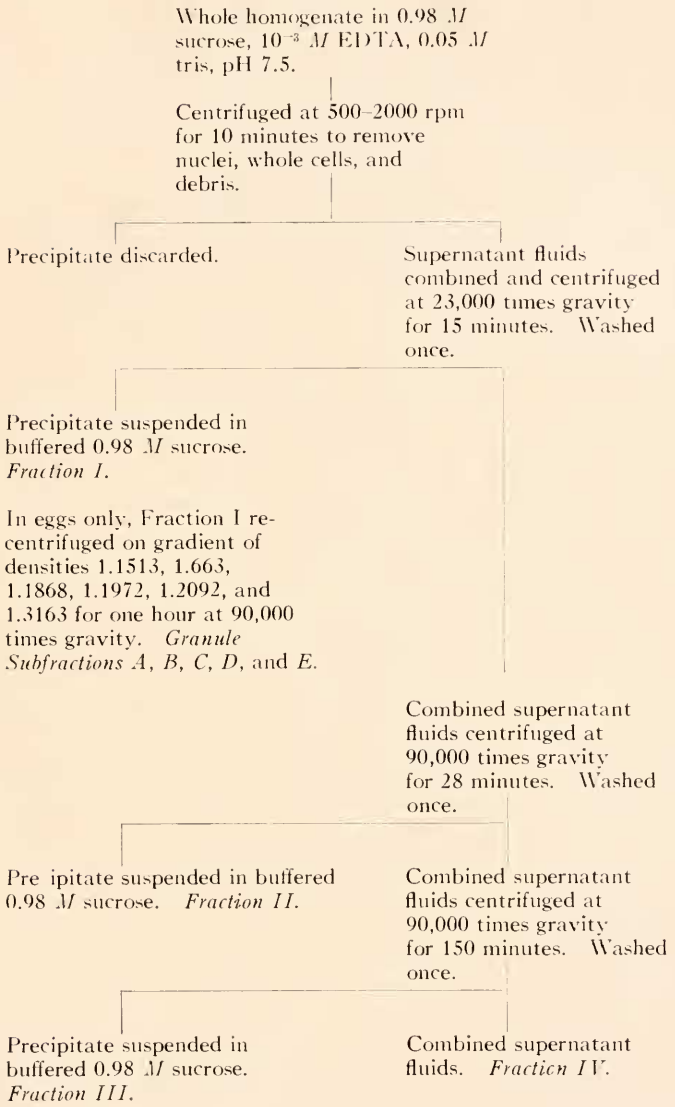
Attempts to measure protease activity at pH 5 were made by the method of Anson (1938), using the liberation of tyrosine from denatured hemoglobin. This method produced extremely variable results with both whole homogenates and subcellular fractions. Whole homogenates gave high blank values, presumably because of a high content of free tyrosine. The assay of *beta*-galactosidase was attempted by the method of Wallenfels (1962), using *p*-nitrophenyl-*beta*-galactoside. No activity could be detected in the whole homogenates, and assays were not performed on subcellular fractions. The assays for lipase, using *p*-nitrophenyl stearate (Huggins and Laprides, 1947), were also negative in whole homogenates of eggs and gut. Attempts to measure succinic dehydrogenase by the ferricyanide reduction method of Bonner (1955) were made only on fresh preparations of Fraction I and its subfractions. This reaction was complicated by the large amount of pigment in this fraction, and it was decided that the data are probably unreliable.

Results

Distribution of enzymes and nucleic acid in subcellular fractions

The distributions of acid phosphatase, esterase, and nucleic acid in the various fractions obtained by differential centrifugation of the gut are shown in Table I. The highest total acid phosphatase and esterase activities are found in the soluble fraction, while the large granules contain only 10 to 15% of the total activity. The microsomal fraction contains the highest specific activity of esterase while in most experiments the specific activity of acid phosphatase was found to be rather uniformly higher in all the granule fractions than in the whole homogenate. The

Outline of Fractionation Procedure



highest per cent of total nucleic acid, as determined by the ultraviolet method, is present in the supernatant fraction, representing very likely mostly soluble ribonucleic acid. The percentage in the microsomal fraction, although slightly higher than in the larger granules, is still quite low, possibly indicating that the cells are poor in ribosomal ribonucleic acid. These findings may be a consequence of the starvation of the animals prior to the fractionation of the tissue.

The distributions of enzyme activities within the subcellular fractions of the eggs are presented in Table II. In addition to the enzymes examined in the gut

TABLE I

Distribution of enzyme activities, nucleic acid and protein in subcellular fractions of adult gut

Specific activity = μM substrate converted/min./mg. protein = factor indicated. Standard errors are given. Number of experiments is indicated after each title.

	Whole	I	II	III	IV	Total %
Acid phosphatase (4)						
Per cent	100	15.5±1.1	8.9±2.0	8.2± 2.6	40.8± 4.3	73.4± 3.2
Sp. act. × 10^5	6.0±0.8	11.9±1.5	11.9±2.0	12.3± 1.2	3.9± 1.2	
Esterase (2)						
Per cent	100	9.3±2.0	8.7±0.0	22.6±11.0	97.6± 0.6	138.2±12.0
Sp. act. × 10^5	1.4±0.0	1.5±0.0	2.0±0.9	4.4± 0.4	1.6± 0.0	
Nucleic acid (2)						
Per cent	100	8.7±2.4	5.2±1.3	10.2± 2.0	70.0± 5.7	94.1±0.2
Sp. amt. × 10^2	2.2±0.0	2.1±0.0	1.4±0.1	2.6± 0.6	1.8± 0.4	
Protein (4)						
Per cent	100	8.2±0.4	4.9±1.7	4.7± 2.2	70.0±11.2	87.8±15.0

subcellular fractions, determinations were made of aryl-sulfatase and RNAase. The highest total acid phosphatase and esterase activities are present in the soluble fractions, but the large granules contain from 25 to 50% of the total acid phosphatase activity and from 15 to 40% of the esterase activity. This difference in percentage of activity present in the large granules of the eggs is probably due to the presence of granules which are not present in the gut. The highest specific activity of esterase is found in the microsomal fraction (III); in this respect the distribution

TABLE II

Distribution of enzyme activities, nucleic acid and protein in subcellular fractions of the egg

Specific activity for RNAase is expressed as μg RNA solubilized/min./μg protein. All other activities are as in Table I.

	Whole	I	II	III	IV	Total %
Acid phosphatase (4)						
Per cent	100	36.1±5.2	11.3±2.9	5.8±0.9	62.3± 7.9	115.5±7.6
Sp. act. × 10^5	3.5±0.4	7.1±0.6	6.8±2.0	13.2±3.9	2.5± 0.5	
Esterase (4)						
Per cent	100	23.2±6.4	12.3±2.8	5.9±0.7	69.2± 3.7	110.6± 7.2
Sp. act. × 10^6	8.3±1.1	11.2±3.3	18.2±3.6	42.3±7.6	9.4± 2.1	
Aryl-sulfatase (3)						
Per cent	100	26.4±3.5	2.1±0.5	0.9±0.4	33.5± 7.2	62.9± 9.0
Sp. act. × 10^4	2.4±0.6	1.9±0.6	0.5±0.1	0.5±0.3	1.4± 0	
RNAase (3)						
Per cent	100	32.1±2.3	17.7±0.5	15.5±1.4	83.6± 7.7	148.9± 9.0
Sp. act. × 10^2	4.7±0.8	4.5±0.8	8.0±2.9	9.8±2.4	8.8± 1.3	
Nucleic acid (3)						
Per cent	100	20.3±1.4	14.2±3.2	32.8±7.0	65.6±12.0	132.9±34.3
Sp. amt. × 10^2	7.7±0.2	4.7±0.7	10.4±0.8	34.4±5.0	11.2± 2.3	
Protein (7)						
Per cent	100	24.3±3.5	7.7±1.2	4.1±1.2	60.3± 6.7	96.4±15.5

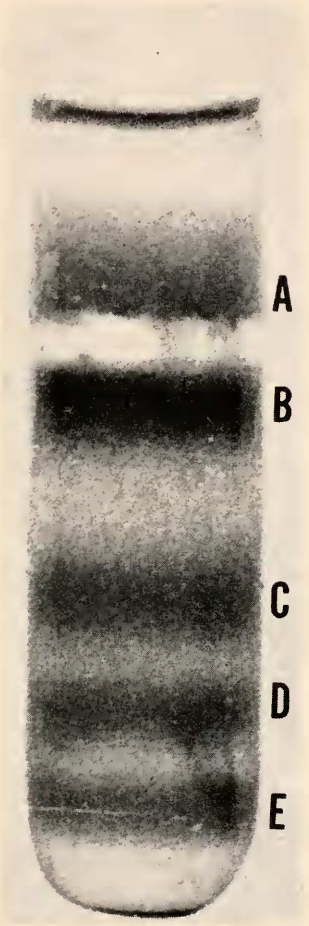


FIGURE 1. Subfractions obtained from Fraction I by density gradient centrifugation. The subfractions were obtained by centrifuging granules on the following densities of sucrose for one hour at 90,000 times gravity: 1.1513, 1.1663, 1.1868, 1.1972, 1.2092, and 1.3163.

is similar to that in the adult intestine. The acid phosphatase in the egg also resembles that of the gut in being rather consistently more concentrated in all granule fractions than in the whole homogenate. The distribution of aryl-sulfatase ranges from 20 to 30% in Fraction I and from 20 to 40% in Fraction IV. The highest specific activity of RNAase, as well as the greatest nucleic acid: protein ratios, were found in the microsomal fraction, Fraction III. The highest percentages of both RNAase activity and total nucleic acid are found in the soluble fraction; however, the recoveries of both these substances are quite high, when the combined amounts in the separated fractions are compared to those in the whole homogenates. It is interesting to note that considerable percentages of the recovered nucleic acid and RNAase were found to be present in the visible granule fraction (I), and in the intermediate granules (II), indicating the probable association of

both substances with non-microsomal particles. This association is examined in more detail below.

Density gradient centrifugation of Fraction I

In order to investigate the possible heterogeneity of the visible granules with respect to their contents of hydrolytic enzymes, Fraction I was further centrifuged in tubes containing several layers of sucrose solutions having different densities. The separation of granules into layers of different densities as a result of this centrifugation is depicted in Figure 1. In most experiments four separable layers were obtained; in one case a fifth, denser layer was also found. Because the centrifugation was performed for only one hour, it seems unlikely that complete separation of granules of different densities was achieved. This incompleteness of separation, as well as a certain amount of mixing which occurred upon removal of the different fractions, undoubtedly contributed to the variations in distribution and activities reported below.

Assays of the hydrolytic enzymes in question, and of nucleic acid were performed on the subfractions obtained by the gradient centrifugation. Tests for DNA

TABLE III

Distribution of enzyme activities, nucleic acid and protein in subfractions derived from fraction I

Specific activities are as in Tables I and II. The letters A through F represent subtractions of different densities obtained by centrifuging Fraction I on a sucrose layer gradient. Subfraction A has the lowest density.

	A	B	C	D	E or F	Total %
Acid phosphatase (3)						
Per cent of Fr. I.	28.0± 0.0	52.3±1.3	12.7±0.4	6.1±0.6		99.1± 1.3
Sp. act. × 10^4	3.4± 0.1	1.1±0.0	0.5±0.0	0.5±0.0		
Acid phosphatase* (2)						
Per cent of Fr. I	51.0±10.0	9.2±2.1	3.2±2.2	3.6±0.3	3.2±0.8 (F)*	70.2± 8.0
Sp. act. × 10^4	0.5± 0.0	0.7±0.1	0.1±0.0	0.3±0.1	2.2±0.2 (F)*	
Esterase (3)						
Per cent of Fr. I.	8.6± 0.1	31.4±0.3	17.0±0.0	1.2±0.6		58.2± 1.0
Sp. act. × 10^5	3.3± 0.1	1.9±0.0	2.1±0.1	0.2±0.1		
Aryl-sulfatase (4)						
Per cent of Fr. I	24.6± 5.2	39.8±5.9	17.8±3.3	15.9±4.6	25.1 (E)**	104.4±15.2
Sp. act. × 10^4	3.0± 0.7	1.7±0.2	2.2±0.4	3.6±0.7	7.2 (E)**	
RNAase (3)						
Per cent of Fr. I	38.8± 3.5	39.8±6.5	35.0±4.0	33.7±2.9	41.8 (E)**	161.3±10.0
Sp. act. × 10^2	8.7± 0.5	3.2±0.8	16.2±4.6	12.0±1.4	26.0 (E)	
Nucleic Acid (4)						
Per cent of Fr. I	13.2+ 1.5	23.7±4.5	18.0±4.2	14.9±3.6	15.9 (E)**	73.3±9.1
Sp. amt. × 10^2	9.1± 1.5	3.8±0.5	8.8±1.8	16.5±4.8	30.0 (E)**	
Protein (8)						
Per cent of W.H.	5.3± 1.4	13.6±1.8	4.7±0.6	2.9±0.5	1.6 (E)**	27.9±2.2
Protein (2)*						
Per cent of W.H.	13.8± 2.4	2.0±0.1	5.9±0.5	1.5±0.3	0.2 (F)*	23.4± 2.2

* Eggs were washed in calcium-free water and Fraction I was centrifuged on the gradient for 90 minutes. Echinochrome granules were concentrated in Layer F.

** Layer E was obtained in only one experiment.

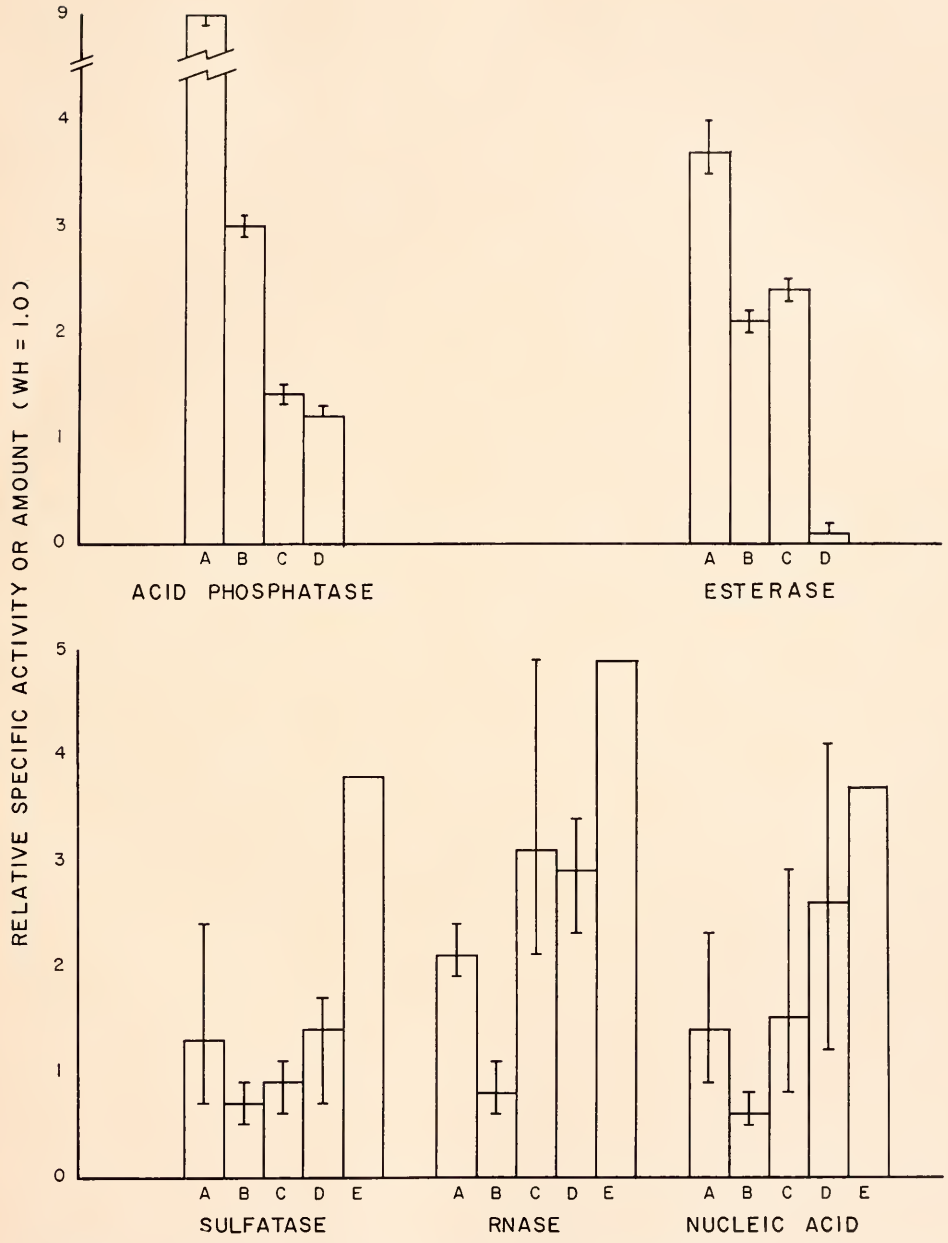


FIGURE 2. Average relative specific activities of enzymes and amounts of nucleic acid in the subfraction granules isolated from Fraction I. The specific activity of the whole homogenate is set at 1.0 for the calculations. The lines represent the range of values for three or four experiments.

were made by the diphenylamine procedure of Dische (1930) as modified by Seibert (1940). These indicated that only trace amounts were present in each fraction. The absorbance of the hot TCA extracts at 260 mμ is therefore tentatively assumed to be a measure of ribonucleic acid content. The results of these determinations are presented in Table III, and the average specific activities of enzymes and amounts of nucleic acid in each granule fraction are shown in a series of histograms in Figure 2. The results indicate considerable heterogeneity in granule types. Acid phosphatase and esterase have the highest specific activities in the granules of lowest density, Subfraction A. Acid phophatase in these granules has a relative specific activity as much as nine times that of the whole homogenate, while esterase is about four times as concentrated as in the whole homogenate. Subfraction B contains the highest percentages of most of the enzymes assayed as well as the highest percentage of total protein. This is assumed to be a result of incomplete separation of granules as noted above. The echinochrome pigment granules are also concentrated in this subfraction in most of the experiments; however, see below. Pigment presumably derived from these granules was always found in the fluid at the top of the density gradient. Aryl-sulfatase, a characteristic lysosomal enzyme, has somewhat higher specific activities in Subfractions A and in the denser granules D and E; however, its distribution is fairly uniform throughout all the subfractions isolated. Ribonuclease activity and nucleic acid are only moderately high in Subfraction A; however, the denser granules, especially D and E, contain unusually high concentrations of both RNAase and nucleic acid.

In two experiments by one of us (R. B.) the eggs were washed several times in calcium-free sea water before homogenization, and Fraction I was centrifuged on the gradient for 90 minutes instead of 60, which resulted in a difference in the distribution of both echinochrome granules and of acid phosphatase. In these experiments the echinochrome granules did not release any pigment, but were driven intact through all the density layers to the bottom of the tube. The granules were evidently in osmotic equilibrium with the 2.5 *M* sucrose, since they immediately ruptured on resuspension in the 0.98 *M* buffered sucrose. The distribution of acid phosphatase in these experiments is also indicated in Table III, with the echinochrome granule layer designated as "F." In these experiments the highest specific activity of acid phosphatase was found to be associated with the echinochrome granules, although the assays were complicated by the presence of the pigment. The echinochrome granule fraction contains a very small percentage of the total protein of Fraction I. The highest percentage of total protein and acid phosphatase was found in Subfraction A in these experiments, while very little protein was present in the B layer. These results appear to indicate that there are at least two types of large, acid phosphatase-containing particles, one of which may be the echinochrome granule.

One of the properties used to define the lysosome is the latency of enzymes in the intact particle (De Duve, 1963). It was of interest to determine whether the activities of acid phosphatase and esterase would be affected if the granules of Subfraction A were subjected to different osmotic conditions. It must be noted that under normal conditions of assay the granules would be ruptured in the dilute reaction mixture. In order to test the effect of osmotic shock, granules of this Subfraction were divided into two aliquots. The first was suspended in 0.05 *M*

tris-10^{-3} *M* EDTA without sucrose, while the other was suspended in buffered sucrose. Assays of acid phosphatase and esterase were performed in the usual manner with the first aliquot, while for the second, all reagents were made up in buffered 0.98 *M* sucrose in an attempt to maintain the granules in an intact state during the reaction. The activity of acid phosphatase was increased 75% by the rupture of the granules, while esterase activity remained unchanged. Treatment with the dilute buffer caused an immediate clarification of the granule suspension. Centrifugation of the clarified suspension at 90,000 *g* for one hour sedimented approximately one-third of the phosphatase and two-thirds of the esterase activity. These results are somewhat inconclusive, since it is obvious that the enzymes of the intact granules are not completely latent. One possibility is that some of the granules are damaged in preparation; however, it is apparent that they still possess a semipermeable membrane. A second possibility is that the enzymes are really not latent within the granules.

Microscopical observation and vital staining of granules

The granules in Subfraction A were observed under oil immersion in order to estimate their size range. The spherical granules varied in size from about one to three microns, with 60–70% being in the 1–1½ micron range, and about 20% in the 2–2½ micron range. Only a very few granules were evident in the 3–3½ micron range, probably less than one or two per cent. No size estimates are available for granules of the heavier subfractions.

The staining of granules from Fraction I, prior to isolation on the sucrose layers, gave conclusive results only with toluidine blue. With this stain Subfractions A and E stained slightly, exhibiting a pale green color. Subfractions B, C and D appeared to exhibit metachromasia to a considerable degree, with most of the red color concentrated in Subfraction B. Neutral red and methyl red were also predominantly taken up by Subfraction B. The presence of red echinochrome pigment granules in this fraction interfered with the detection of its staining properties with all dyes. It seems clear that the granules of Subfraction A, which possess the highest specific activity of acid phosphatase, do not stain metachromatically *in vitro*. A direct analysis of the nature and content of polysaccharides in the different granules would appear to be desirable from the standpoint of correlating this finding with that of Dalcq (1963), who reported that granules possessing acid phosphatase activity also exhibited metachromatic properties.

Discussion

From the results illustrated in Figure 2, it may be tentatively concluded that at least two types of visible granules, differing in their content of hydrolytic enzymes, exist in the egg. The first type, exemplified by Subfraction A, exhibits acid phosphatase and esterase activities. The second type, found in Subfractions D and E, contains RNAase and nucleic acid, as well as a relatively high content of arylsulfatase.

The granules of Subfraction A contain at least two of the hydrolytic enzymes believed by de Duve (1963), Novikoff (1961) and others to be located within the lysosomes. The absence of metachromasia in these granules after *in vitro* staining

suggests that they may differ from the *alpha* and *beta* granules of Dalcq (1963) and Pasteels and Mulnard (1957) in their polysaccharide content; however, these investigators worked only with fertilized eggs stained *in vivo*. Rebhun (1959) demonstrated that in *Spisula solidissima* staining of the *alpha* and *beta* granules appeared only after fertilization. In stratifying eggs of various species of sea urchins, Immers (1960) expressed doubt that regions of mucopolysaccharide concentration evident after *in vivo* staining corresponded to the metachromatic *alpha* and *beta* granules of Dalcq and Pasteels because his staining was performed only on unfertilized eggs.

In spite of their high content of hydrolytic enzymes, it would be premature to identify the granules of Subfraction A as lysosomes. De Duve (1963) has warned that the present definition of the lysosome, although based primarily on his rat liver tissue work, must not include any incidental details such as size and other physical characters, osmotic properties, centrifugal behavior, mechanism of structure-linked latency, or sensitivity to individual disrupting treatments. If these factors are therefore omitted in defining the lysosome, the essential characteristic remaining is the association within a special group of cytoplasmic particles of a number of soluble acid hydrolases of widely differing specificity. The accessibility of these enzymes to the surrounding substrate must be restricted, making the latency of the enzymes dependent on the structural complexes of the particles. Such a definition would be broad enough to include the hydrolytic granules in Subfraction A isolated from *Arbacia* eggs, if it could be shown more conclusively that the accesssibility or activity of the enzymes in question is restricted by the granular structure.

The finding that acid phosphatase activity is associated with the echinochrome pigment granules is of considerable interest. Since these granules were ruptured by dilution after recovery from the 2.5 *M* sucrose layer, the effects of different osmotic treatments were not tested. Further experiments on these granules are in progress.

We believe that the mitochondria are concentrated in Subfraction B, since it contains the highest percentage and specific activity of succinic dehydrogenase; however, the data for this enzyme appear to be rather unreliable. This subfraction is probably heterogeneous, since it contains the highest percentages of all enzymes and of total protein.

The most dense granules in the visible granule fraction, recovered in Subfractions D and E after gradient centrifugation, contain much higher specific concentrations of nucleic acid and RNAase than do the other visible granules. In preliminary experiments we have made determinations of amino acid incorporation into protein of these heavy granules after giving unfertilized eggs a 10-minute pulse with C^{14}-phenylalanine. After such a pulse, the specific activity of Subfractions D and E, calculated on the basis of nucleic acid content, is only about one-tenth that of the microsome fraction; these granules are therefore quite inactive in protein synthesis, even in the fertilized egg. We have concluded that there is little contamination from microsomes in this fraction. The existence of dense RNA bodies in eggs has been reported by other workers. Raven (1945) demonstrated the presence of heavy RNA particles in the centrifugal pole of stratified *Limnaea* eggs. Pasteels (1958), by centrifuging *Paracentrotus* eggs, discovered "heavy bodies" of

RNA, ranging from 1–3 microns, in the centrifugal cap region. This region, which also contained the mitochondria, was intensely stained with pyronine. Pasteels postulated that, in addition to being found in the ribosomes and in annulate membranes within the egg, RNA could also be found in undefined structures that could be linked to the mitochondria but which contained the most dense material in the egg. Balinsky and Devis (1963) observed electron-dense granules in the young oocytes of *Xenopus laevis* which presumably accumulated between adjacent mitochondria. Afzelius (1956) has also described "heavy bodies" which stain vitally with toluidine blue in the sea urchin egg. Immers (1960) described dense RNA granules which were separate from the mitochondria in the most centrifugal zone of stratified eggs of *Paracentrotus lividus*. A few workers have claimed that the heavy yolk granules, especially in the Amphibia, contain an appreciable amount of RNA (Grant, 1953; Rounds and Flickinger, 1958), but others have shown by histochemical and cytological studies that there is little or no RNA within the yolk granules of most species examined. Collier (1960) found no evidence of either RNA or proteolytic enzymes in the yolk granules of *Ilyanassa obsoleta*. The recent work by Karasaki (1963) and Ohno *et al.* (1963) revealed no evidence for the presence of RNA in the yolk granules of *Triturus pyrrhogaster* and *Rana pipiens* embryos.

It may be tentatively concluded that the heavy granules in Subfractions D and E correspond to those described by Immers and Pasteels. It is possible that the annulate lamellae described by Pasteels (1958) are sufficiently dense to be included in this fraction; these structures consist of membranes to which bodies similar in size and density to ribosomes are attached. No previous report has been made concerning the association of RNAase with any large granule fraction in the egg; however, a comparison of the present finding with that of Reid and Node (1959) for granules of rat liver is of particular interest. These authors provided evidence that acid RNAase was present in particles which were more rapidly sedimented from homogenates than the lysosomes, indicating the possible existence of a separate set of granules which contain this enzyme.

The distribution of activities of the hydrolytic enzymes in Fractions II and III of the egg homogenates indicates the presence of these enzymes in submicroscopic structures (see Table II). The possibility therefore exists that granules resembling rat liver lysosomes in size may also be present in the egg. If such granules are easily ruptured during preparation, as are liver lysosomes, this may account for the high enzyme activities found in the soluble fraction. It is of interest that Fractions II and III of the gut tissue of adult *Arbacia* (Table I) contain approximately the same specific activities of acid phosphatase and esterase as do the corresponding fractions of the egg.

The heterogeneity of the populations of granules which contain hydrolytic enzymes in the egg may be generally related to the timing with which different enzymes become active during development. It is postulated that such a separation of enzymes in different granules could result in the specific release or activation of some hydrolases, but not others, at particular developmental stages. Furthermore, partial segregation of the granules into different cells during cleavage may confer different developmental potentialities on the daughter cells. Segregation of granules and certain enzymes have been observed in numerous eggs exhibiting "mosaic"

cleavage (*cf.* Brachet, 1950, for review); however, similar differentiation has not been observed in the sea urchin. Experimental testing of the latter hypothesis must await the development of techniques for visual identification of the granules in question.

Summary

Differential centrifugation and density gradient centrifugation have been applied to nuclei-free homogenates of unfertilized eggs and adult gut of *Arbacia*, to determine the distributions of several hydrolytic enzymes and of nucleic acid and protein. Two types of large visible granules have been partially separated from egg homogenates by gradient centrifugation. The first type is rich in acid phosphatase and esterase; the second contains sulfatase, RNAase and nucleic acid. The activities of the above enzymes have also been determined in microsomal and soluble fractions of the egg, and the distribution of acid phosphatase and esterase have also been determined in the major subcellular fractions of the adult gut of *Arbacia*. In both types of homogenates hydrolytic enzymes were found to be present in submicroscopic granules and in the supernatant fluid. The major difference in the two types of material is that large granules containing the enzymes are present in the eggs but not in the gut tissue.

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